DNA Adducts as a Dosimeter for Risk Estimation

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The dose response for O^6-methylguanine (O^6MG) formation and cytotoxicity was determined in lung and nasal mucosa from Fischer 344 rats during multiple dose administration of the tobacco-specific nitrosamine 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK). O^6MG accumulated in the lung following treatment for 12 days with doses of NNK from 0.3 to 100 mg/kg/day. The dose response for NNK was nonlinear; the O^6MG-to-dose ratio, an index of alkylation efficiency, increased dramatically as the dose of carcinogen decreased. These data suggest that low- and high-K_m pathways may exist for activation of NNK to a methylating agent. Marked differences in O^6MG concentration were observed in specific lung cell populations. The Clara cell, one of the suggested progenitor cells for nitrosamine-induced neoplasia, was found to possess the greatest concentration of O^6MG. Moreover, as the dose of NNK was decreased from 100 to 0.3 mg/kg, the alkylation efficiency in this cell population increased 38-fold. The presence of a high-affinity pathway in the Clara cell for activation of NNK may contribute to the potent carcinogenicity observed following low-dose exposure to this tobacco-specific carcinogen. The dose response for O^6MG formation differed considerably between the respiratory and olfactory mucosa from the nasal passages of the rat. The dose response was nonlinear in respiratory mucosa but linear in olfactory mucosa. The alkylation efficiency increased dramatically only in the respiratory mucosa as the dose of NNK was decreased. These studies suggest that a low K_m pathway for NNK activation is also present in the nose and that this pathway is localized predominantly in the respiratory region. In contrast to alkylation data, greater sensitivity to toxicity by NNK was observed in the olfactory region. Only mild metaplasia and a 5% incidence of malignant tumors were observed in the respiratory region. However, treatment with NNK resulted in marked necrosis of the Bowman's glands and olfactory epithelium leading initially to hyperplasia and basal cell metaplasia and finally to a 45% incidence of malignant tumors in this area of the nose. These data suggest that both the formation of promutagenic adducts and cell proliferation secondary to toxicity are required for the induction of neoplasia by NNK within the nose.

Introduction

One of the major shortcomings in estimating carcinogenic risk from long-term exposure to chemical carcinogens is the uncertainty of extrapolating from high to low doses. The majority of carcinogenicity studies encompass a dose range of less than one order of magnitude and in some studies the dose chosen is based on the maximum tolerable dose established from acute toxicity experiments. After completion of the bioassay study, estimation of tumor incidence from low-dose exposure may require data extrapolation over one to two orders of magnitude (Fig. 1). This in turn may lead to either over- or underestimation of the carcinogenic potency of many chemicals. Recent studies (1) have suggested that for chemicals with metabolites that interact with DNA, it may be more meaningful to relate tumor response to the target organ concentration of DNA adducts than to the applied dose. However, this model
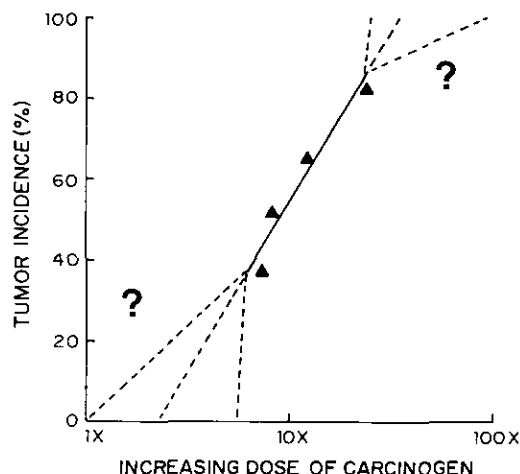

FIGURE 1. Possible consequences of high-dose extrapolation from tumor incidence data. Hypothetical data illustrating the problems in extrapolating to low doses using tumor incidence from high-dose exposure.

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may also be too simple to ensure accurate risk esti-
mation. For example, the concentration of DNA adducts
may vary greatly within the vast cellular heterogeneity
of tissues such as lung and kidney. There are also sev-
eral factors that may ultimately influence the initiation
of cell transformation by an environmental chemical
(Fig. 2). The electrophilic intermediate generated fol-
lowing metabolic activation of a compound can either
bind covalently to DNA, RNA, or cellular proteins, or
be removed from the cell via detoxification pathways.
Covalent binding to DNA will not always result in fix-
ation of promutagenic damage if the damaged template
is repaired before cell replication has been initiated.
Conversely, for some chemicals the efficiency for fixa-
tion of promutagenic damage may be enhanced if con-
stitutive levels of cell turnover are increased via toxicity
or the presence of promoters such as phenobarbital.
Thus, there are many factors that must be considered
to allow more accurate estimation of the tumorigenic
potential of an environmental agent.

During the past 5 years, several laboratories (2–6)
have begun to examine the dosimetry of DNA adduct
formation and cytotoxicity during multiple-dose expo-
sure to carcinogens. Our laboratory has been interested
in understanding the tissue selectivity of the tobacco-
specific nitrosamine 4-(N-methyl-N-nitrosamino)-1-(3-
pyridyl)-1-butanone (NNK). In rodent carcinogenicity
studies (7) treatment with high doses of NNK resulted
in a similar high incidence of lung, liver, and nasal cavity
tumors, whereas after lower doses of NNK, the prev-
alence of malignant tumors was much greater in lung
than in other target tissues. Specifically, the incidence
of malignant tumors in the nasal passages decreased
from 70% to less than 5% as the dose of NNK was
reduced from 50 to 5 mg/kg (treatment was for 20
weeks, three times a week), while the 50% incidence of
malignant neoplasia observed in the lung was unaffected
after decreasing the dose of carcinogen administered.
The purpose of this study will be to determine whether
DNA alkylation and cytotoxicity data can explain the
differences in the dose response for induction of neo-
plasia by NNK in the nasal cavity and lung.

Cell Selective Alkylation of DNA in the Lung

The potent carcinogenicity of NNK may stem in part
from its metabolic activation via α-hydroxylation to a
methylating agent (8). Administration of high doses of
NNK (100 mg/kg/day) for 12 days to Fischer 344 rats
resulted in the accumulation and persistence of the pro-
mutagenic adduct O^6MG in lung (2). The accumulation
and persistence of this adduct correlated with the deple-
tion of the repair protein O^6-methylguanine-DNA meth-
yltransferase. These studies demonstrated that a pro-
mutagenic lesion accumulates in lung during exposure
to NNK. However, estimation of the carcinogenic risk
from this high-dose study (2) suffers from the problem
of high- to low-dose extrapolation. Moreover, due to the
cellular heterogeneity of the lung, the accumulation of
DNA adducts may vary greatly among specific pulmo-
nary cell populations. Therefore, the dose response for
O^6MG formation in DNA from lung and specific cell
populations isolated from lung was determined during
multiple administrations of NNK.

O^6MG accumulated with doses of NNK ranging from
0.1 to 100 mg/kg/day throughout the 12 days of carcin-
ogen administration (3). A plot of adduct concentrations
as a function of dose after 12 days of treatment revealed
that the relationship between dose and O^6MG accu-
mulation was nonlinear (Fig. 3A). The slope of the line
was very steep with doses of NNK from 0.1 to 3 mg/
kg, but declined markedly in the dose range of 10 to
100 mg/kg. The concentration of O^6MG formed to dose
of NNK ratio, an index of efficiency of alkylation,
increased dramatically from 0.6 to 11.4 as the dose of
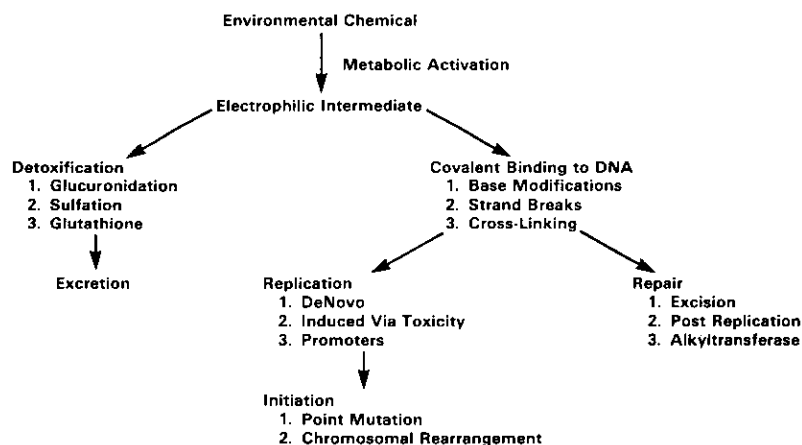NNK was decreased from 100 to 0.1 mg/kg/day (Fig.

FIGURE 2.   Factors that can influence the initiation of cell transformation. A flow diagram illustrating the complexity of the process of chemically induced cell initiation.
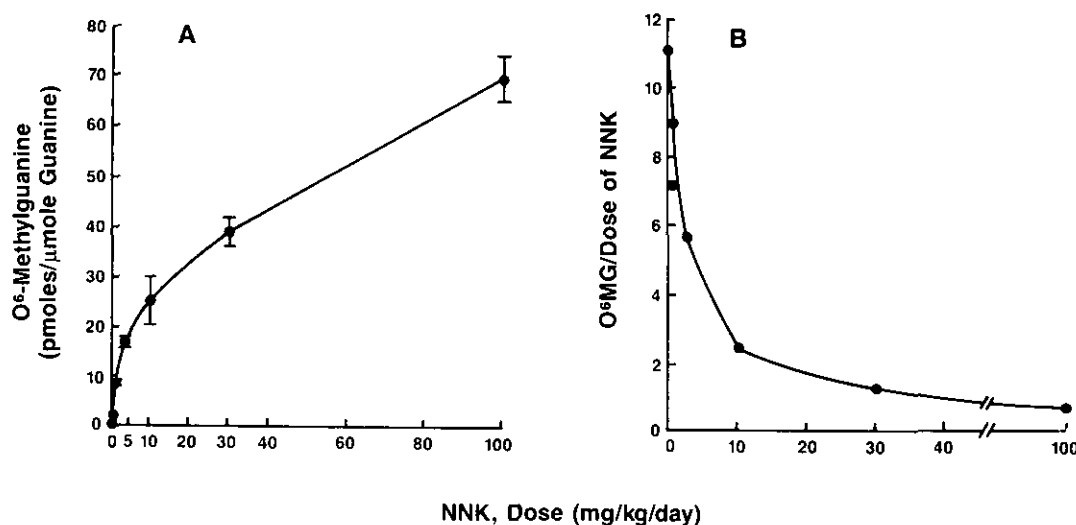
NNK, Dose (mg/kg/day)

FIGURE 3.   Dose response (A) and efficiency of O^6-methylguanine formation (B) in lung. (A) O^6-methylguanine was determined in lungs from rats treated for 12 days with 0.1, 0.3, 1, 3, 10, 30 or 100 mg/kg NNK, as described in (3). Points are means from four rats; bars, SE. (B) Concentration of O^6MG (pmole O^6MG/μmole guanine) in lungs after 12 days of treatment with NNK was divided by the dose of carcinogen (mg/kg/day) and plotted against dose.

3B). These studies demonstrate that the efficiency for O^6MG formation from NNK is much greater after low than high doses of carcinogens. Therefore, the extrapolation of carcinogenic risk from NNK using high-dose alkylation data would greatly underestimate risk.

However, data only from the target organ may be insufficient for accurate risk assessment. The Clara cell, although accounting for only 1% of the pulmonary cells in the lung of the rat, was found to possess the highest concentration of O^6MG following treatment with NNK (Table 1). Moreover, as the dose of NNK was decreased from 100 to 0.3 mg/kg, the alkylation efficiency in this

Table 1. Predominance of O^6-methylguanine in Clara cells following multiple doses of NNK.[a]

| Cell type | pmole O^6MG/μmole guanine | | | |
| | NNK treatment, mg/kg/day | | Alkylation efficiency, O^6MG/dose, mg/kg/day | |
	0.3	100	0.3	100
Whole lung	0.9 ± 0.2	43 ± 3	3.0	0.4
Alveolar small cells	1.5 ± 0.7	40	5.0	0.4
Alveolar macrophages	3.8 ± 0.6*	173 ± 7*	12.7	1.7
Type II cells	1.1 ± 0.2	44 ± 4	3.7	0.4
Clara cells[b]	28.2 ± 1.7*	254 ± 8*	93.2	2.5

[a] Rats were treated for 4 days with either 0.3 or 100 mg/kg NNK. Animals were sacrificed 4 hr after treatment on day 4. Lung cells were pooled and isolated from 6–12 rats, and DNA was isolated for determination of O^6MG by competitive radioimmunoassay as described in (3). Alkylation efficiency was determined by dividing the concentration of O^6MG by dose of NNK. Values are means ± SE from 3 to 4 sets of animals.
[b] Calculated levels of O^6MG in Clara cells, excluding contamination from other cell types.
*Significant difference (p < 0.01) when compared to whole lung.

cell population increased 38-fold. This was in contrast to the eightfold increase in alkylation efficiency observed in lung.

The decrease in alkylation efficiency during exposure to high doses of NNK probably cannot be explained by cytotoxicity, since treatment of rats for 12 days with NNK (100 mg/kg/day) caused no alterations in cell morphology based on histological examination (2). Also, one would not expect the complex kinetics of methylation in the lung to be influenced by hepatic metabolism because the dose response for alkylation in the liver was linear (S. A. Belinsky, unpublished data), indicating that the effective dose of NNK reaching the lung should be proportional to the injected dose. The high efficiency for O^6MG formation following low-dose exposure to NNK may stem from the presence of multiple pathways for activation of this carcinogen in the lung. The metabolism of NNK to a methylating agent is thought to occur by cytochrome P-450-dependent α-hydroxylation (8). Recently, several different cytochrome P-450 isozymes have been identified in the rat lung (9). Based on the two-component nature of the alkylation efficiency curve (Fig. 3), we hypothesize that low and high K_m pathways exist for the activation of NNK to a methylating agent in the lung. This hypothesis is supported by both autoradiographic studies and alkylation data. Autoradiographic grains were more heavily concentrated over Clara cells than over other cell types in the lung after treatment of rats with 1 mg/kg NNK (3). In addition, the increase in alkylation efficiency, which occurred as the dose of NNK was decreased, was much greater in Clara cells than in other pulmonary cell types. Taken together, these data suggest that a low K_m pathway for activation of this tobacco-specific carcinogen is more
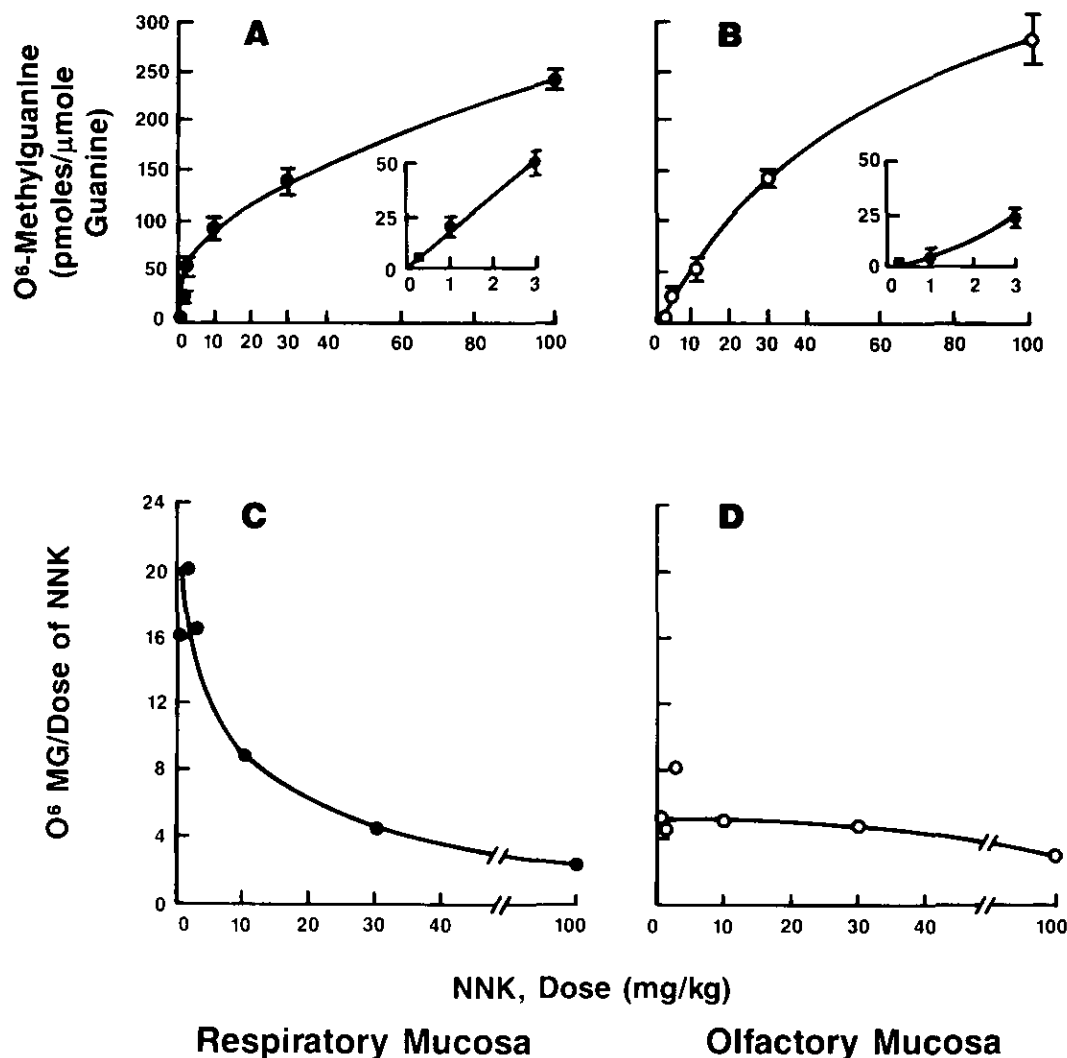
FIGURE 4. Dose response (A,B) and efficiency of O^6-methylguanine formation (C,D) in nasal mucosa. O^6-methylguanine was determined in respiratory (A) and olfactory (B) mucosa from rats 4 hr after treatment with 0.3, 1, 3, 10, 30 and 100 mg/kg NNK as described in (10). The concentration of O^6MG (pmole/μmole guanine) in respiratory (C) and olfactory (D) mucosa 4 hr after treatment with NNK was divided by the dose of carcinogen (mg/kg) and plotted against dose.

concentrated within the Clara cell. Thus, the presence of a high-affinity pathway in the Clara cell for activation of NNK may be an important factor in the carcinogenicity of this tobacco-specific nitrosamine following low-dose chronic exposure.

Cell Selectivity for DNA Alkylation and Cytotoxicity in the Nasal Passages

During multiple administrations of NNK (2), DNA alkylation was also detected in the nasal passages and liver from the Fischer rat. In fact, after 1 day of treatment with NNK (100 mg/kg), the concentration of O^6MG was greatest in nasal mucosa, followed by concentrations of O^6MG in the liver and lung (2). However, during the next 5 days of treatment, alkylation of DNA in nasal

epithelium declined to one-half the initial amount and remained constant for the remaining 6 days of carcinogen administration. The decrease in O^6MG concentration appeared to be the result of cytotoxicity induced by exposure to NNK. To better understand the relationship between DNA damage and cytotoxicity in the induction of neoplasia in the nose by NNK, the dose response for these biological end points was established.

The dose response for O^6MG formation differed considerably between respiratory and olfactory mucosa. The concentration of O^6MG was two to four times greater in respiratory than olfactory mucosa after treatment with doses of NNK ranging from 0.3 to 3.0 mg/kg and 60 to 90% higher during multiple dose administration (10). The dose response was nonlinear in respiratory mucosa where the slope of the curve was very large for doses of NNK ranging from 0.3 to 3.0 mg/kg, but much smaller in the dose range of 10 to 100 mg/kg (Fig. 4A).

In contrast, the dose response in the olfactory mucosa did not demonstrate such a large change in slope over the same dose range (Fig. 4B). The alkylation efficiency increased dramatically only in the respiratory mucosa as the dose of NNK was decreased from 100 to 0.3 mg/kg (Fig. 4C,D). The difference in the shape of the dose-response curves for O^6MG in respiratory and olfactory mucosa may stem from the localization of metabolic pathways for biotransformation of NNK. The amount of O^6MG formed per unit dose of NNK increased eight-fold in respiratory mucosa as the dose of carcinogen was decreased from 100 to 0.3 mg/kg (Fig. 4). A similar alkylation efficiency curve for O^6MG has also been observed in rat lung after treatment with NNK (Fig. 3). The fact that the concentration of O^6MG was greater in respiratory than olfactory mucosa and that the efficiency for alkylation increased only in respiratory mucosa after low-dose exposure suggests that a low K_m pathway for NNK activation is also present in the nose and that this pathway is localized predominantly in the respiratory region. This conclusion is supported by autoradiographic studies that demonstrated that silver grains were more heavily concentrated over respiratory than olfactory epithelium (10) 4 hr after treatment of rats with 1 mg/kg NNK.

Because DNA isolated from respiratory mucosa is selectively alkylated after low doses of NNK, one would hypothesize that this region would also exhibit greater sensitivity for cytotoxicity and neoplasia than the olfactory mucosa following carcinogen exposure. However, no toxicity was observed in either portion of the nasal passages (10) with the dosing regimen that gave the largest difference in alkylation between the two regions of the nose (1 mg/kg). Following exposure to higher doses of NNK, the respiratory mucosa and underlying glands were more resistant to toxicity and neoplasia, exhibiting only mild metaplasia and a 5% incidence of malignant tumors. In contrast, treatment with NNK resulted in marked necrosis of the Bowman's glands and olfactory epithelium, leading initially to hyperplasia and basal cell metaplasia, and finally to a 45% incidence of malignant tumors in this area of the nose (10). Based on autoradiography and alkylation studies, it is apparent that the differential toxicity to the nasal passages induced by NNK cannot be attributed to either the local concentration of DNA adducts or to the NNK itself. At present, the mechanism for this selective toxicity is not understood.

These experiments indicate that adduct concentration in the nose alone may not be a good index of carcinogenic risk from NNK. Based solely on the alkylation efficiency curves for O^6MG (Fig. 4), one would conclude that the probability for initiation of tumorigenesis should be much greater in respiratory than olfactory regions after low-dose exposure to NNK. However, the majority of malignant tumors induced by NNK appear to originate from the olfactory region and occur only after chronic treatment with high doses of NNK (16 or 50 mg/kg). The steep dose-response curve for induction of tumors (7), as well as the localization of lesions in the nasal

passages, can be explained by a difference in sensitivity to cytotoxicity by NNK. Although the concentration of O^6MG was greater in respiratory regions after low-dose exposure to NNK, no cytotoxicity was observed in either region of the nose. In contrast, after exposure to high doses of the carcinogen, adduct concentrations were similar in both regions of the nose; however, marked cytotoxicity was localized to the olfactory region. Cell proliferation in the control Fischer 344 rat is very low (0.1 to 0.2%) in both respiratory and olfactory epithelium (11). Therefore, a marked increase in cell proliferation in the olfactory region demonstrated by severe basal cell metaplasia secondary to NNK-induced necrosis could result in mutation at the site of DNA adducts, clonal expansion of initiated cells, and a much greater probability of cells undergoing subsequent critical mutations. Taken together, these data indicate that both the formation of promutagenic adducts and cell proliferation secondary to toxicity are required for the induction of neoplasia by NNK within the nose.

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